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14. ABSTRACT
The so-called "lethal phenotype" in prostate cancer (CaP) involves the recurrence of metastatic disease after androgen-ablation therapy. However, little is known regarding the genetics that control disease recurrence. Our proposed research was to screen for metastasis-inducing genes in LNCaP and LAPC-4 CaP cells using libraries expressing RNAi covering the entire human genome. We have infected the CaP cell lines with two libraries, a retrovirus library encoding validated shRNAs for 983 "cancer genes" and a lentivirus genomic shRNA library (GIPZ). We have identified cells with significantly increased capabilities for Matrigel invasion over spontaneous rates, and we are now identifying and validating individual genes whose knockdown contributes to the increased invasive potential of CaP cells. In addition to potentially serving as clinically predictive biomarkers, these genes will be useful in understanding the molecular events required for metastatic progression in CaP.

15. SUBJECT TERMS
Prostate cancer, metastasis, invasiveness, shRNA, RNAi, genomic libraries, LNCaP, LAPC-4, retrovirus, lentivirus, Matrigel, bone colonization.

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Table of Contents

INTRODUCTION

We propose to use a novel and revolutionary technology based on so-called RNA-interference (RNAi), in which specific genes can be turned off using tailored RNAi molecules. The field of RNAi is only 5-6 years old, and the PI is the Director of a CORE Facility at RPCI that houses one of the first RNAi libraries covering all known human genes (~35,000 genes). Thus, we propose to turn off individual genes in a population of human CaP cells by treating them with an entire human genomic RNAi library. We will then select for those cells that have significantly increased metastatic behavior. The three growth parameters we will test are increased ability to i) invade tissues, ii) colonize the bone, and iii) grow as metastatic bone lesions. After several rounds of selection to enrich for more metastatic cells, we will be able to identify which genes were turned off using gene-specific "bar-code" sequences engineered into the RNAi molecules. We will then use molecular probes for these genes or their protein products to determine whether there is a correlation between their being turned off and an increasing metastatic potential in either cultured CaP cell lines or in human CaP tissues. Such a finding would lay the foundation for future, more in-depth studies to show that the loss of these gene products could be a predictor for metastatic disease. In addition, future hypothesis-driven studies would address how these genes normally prevent progression to the metastatic phase, thereby affording a greater understanding of the molecular mechanisms governing CaP progression. Continued study of these genes would also contribute to an understanding of how CaP cells colonize and grow in the bone environment- a field that is critical to understanding CaP metastatic disease yet for which there is little data regarding genetics.

BODY

Below are each of the tasks approved in the Statement of Work followed by a report on how the tasks aims were met (or not), or how experiments were adapted to address the intent of the tasks (in bold)

Task 1. Transduce LNCaP and LAPC-4 with RFP (Months 1-3).

-culture LNCaP and LAPC-4 cells as "targets". Transfect 29GPG retrovirus packaging cells with pBABE/RFP/*hygro*. Use transiently expressed virus to infect target cells, select RFP-positive cells in hygromycin-containing media. If need be, high RFP expressors can be sorted by FACS®.

Sets of LNCaP and LAPC-4 were transduced with RFP successfully. Because our shRNA CORE Facility (the PI is the facility's Director) was able to procure the human genomic shRNA GIPZ lentivirus library, where each clone expresses an IRES- GFP cassette (i.e.- labeling all transduced cells with GFP), we also used the GFP-labeled shRNA-expressing cells in a parallel analysis. Note that we delayed the project for almost 6 months in order for Open Biosystems LLC to provide us with the GIPZ library (the company had quality-control complications that delayed the release). We felt this delay was appropriate because this library had several distinct advantages: increased numbers of functionally-validated shRNA clones, the internal IRES-GFP marker, and high virus titers. Lastly, this library was controlled for shRNA clone numbers, specifically, that it contained >95% of all the library's clones as shown by hybridization to arrays containing anti-sense bar-code oligonucleotides. The pSM2 library that we were producing in our shRNA CORE Facility could not be validated in such as fashion because Agilent decided not to produce the anti-sense bar-code arrays commercially.

Task 2. Produce human genome-wide shRNA retrovirus library. (Produced by RPCI shRNA CORE Facility prior to the onset of the project).

-grow 25 aliquots each of 3000 shRNA retrovirus clones, isolated plasmid DNA. Transfect 25 aliquots of 293GPG packaging cells, harvest transiently expressed viruses on day 2, titer virus for puromycin-resistant colony-forming activity units (must be $>10^5$/ml).

Again, because of the availability of the GIPZ shRNA library, we only produced the retrovirus, pSM2-based library, using the so-called Cancer Gene library (also provided by Open Biosystems, LLC). This library contains roughly 1200 functionally-validated clones that cover 983 human cancer-related genes such as kinases, oncogenes and tumor suppressors. We produced viruses from this library in a total of three aliquots of clones (ca. 400 clones/aliquot), and used these to infect RFP-expressing LNCaP and LAPC-4 cells.

Task 3. Infect LNCaP/RFP and LAPC-4/RFP cells. (Months 3-5).
-infect target cells and then select for growth on puromycin-containing media. Pool colonies, expand.

The RFP cells were infected in triplicate, selected for puromycin-resistant colonies (multiplicity of infection was roughly 0.5), and the cells were expanded. The titers of the concentrated GIPZ virus aliquots were >7 x 10^8 GFP-forming units/ml, and thus, we were able to infect these cells at MOIs >1 such that >98% of all cells expressed GFP.

Task 4. Select for increased Matrigel invasiveness. (Months 5-12).
-subject populations of cells to 6 h migration assays in Matrigel-coated transwells. Note: concentrations and thicknesses of Matrigel will be used that show minimal invasiveness of the parent cells. The pool of shRNA-infected LNCaP/RFP and LAPC-4/RFP cells will be divided into triplicates of 24-well assays in order to maximize the isolation of individual clones with increased invasiveness. Scape off and expand the RFP-positive invasive cells. Repeat the selection process at least twice more in order to isolate cells with increased invasive potentials over parental cells.

As shown in Fig. 1, the background of spontaneously-invading LNCaP cells was quite low, typically less than 0.02% (i.e.- roughly 100 invading from 5 x 10^5 seeded cells above the Matrigel). Expansion of these cells (which took several weeks because of the small number of invading cells isolated after the first round) was completed, and the invasion assay/cell harvesting was performed for two more rounds. Fig. 1 shows that although there was a selection for control CaP cells with increased invasive potential, the shRNA-transduced cells exhibited significant increases for the selection of invasive cells. This strongly suggests that specific gene knockdowns contribute to the increased invasive potential of the shRNA-transduced populations. We are currently in the process of cloning the invasive cells, retesting individual clones for increased invasiveness (*Task 7*), and then identifying the shRNA-mediated gene knockdowns by bar code PCR (*Task 8*).

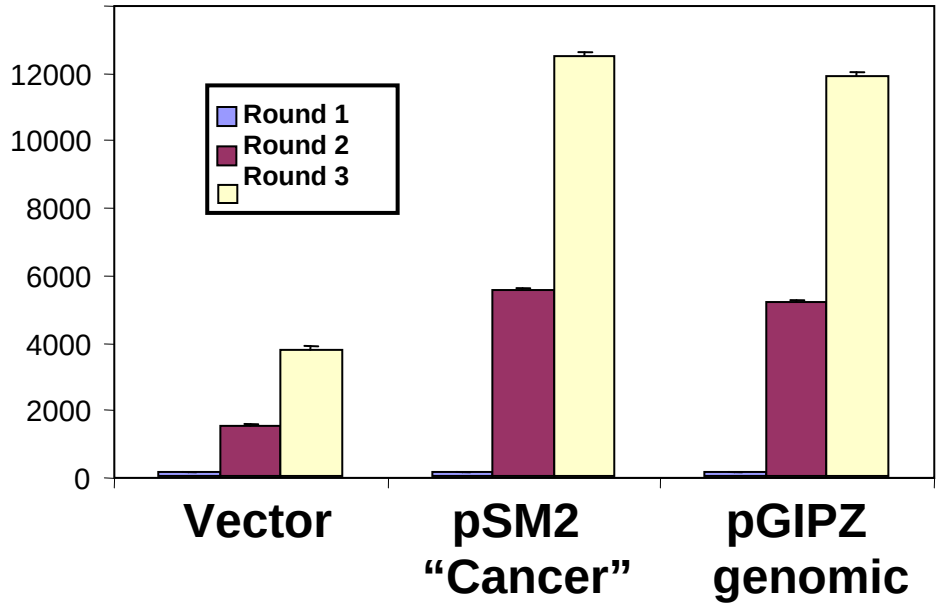

Figure 1. Enrichment for LNCaP cells with increased invasive potential. LNCaP transduced with empty vector, the retrovirus "Cancer Gene" shRNA library in pSM2, or the human genomic shRNA lentivirus library (GIPZ) were subjected to triplicate assays of Matrigel invasiveness, using 5 x 10^6 cells/well in 6-well dishes. The number of cells that invaded in 6-h assays after 1, 2 or 3 rounds of selection is shown in the Y-axis. Error bars, S.E.

Task 5. Select for increased bone colonization. (Months 6-13).

-inject at least 15 nude mice in their left ventricles (i.c.) with ~10^6 cells/mouse of shRNA-infected LNCaP/RFP or LAPC-4/RFP cells. Inject a similar number of mice with mock-infected LNCaP/RFP and LAPC-4/RFP cells. Monitor bone colonization 3 days later by sacrificing the mice, and then pooling scraped bone marrow plus collagenase-treated single-cell suspensions of bone (femurs). Subject half the samples to FACS® analysis and determine the number of RFP-positive cells per 10^6 total bone cells; monitor for increased colonization over control cells. Culture the remaining cells in hygro/puromycin-containing media to select for the CaP cells, expand. Re-select for increased bone colonization at least one more round.

We are currently pursuing this aim. Our work will be aided by the formation of a new core lab at RPCI, namely the Mouse Tumor Model Core Resource (Barbara Foster, Director). This facility has technicians who are expert in intracardiac injections.

Task 6. Select for increased bone metastatic growth. (Months 7-14).

-Follow the protocol in *Task 5*, except that the end-point is the growth of boney metastases. Reduce number of mice injected with the parental CaP cells to 6 each (since they are poor inducers of bone metastases after i.c. injection). Monitor (weekly) bone metastasis formation using a Faxitron rodent X-ray instrument for a total of 6-7 months. After sacrificing animals, remove the bone tumors and expand the CaP cells in culture.

We are currently pursuing this aim.

Task 7. Single-cell clone the cell variant with increased metastatic potential. (Months 12-15). -use terminal dilution techniques to clone metastatic cells.

We are currently pursuing this aim.

Task 8. Identify RNAi-mediated gene knockdowns. (Months 12-16).

-Expand shRNA-specific "bar-code" sequences by PCR; analyze the PCR products by DNA sequencing. Verify by q-RT-PCR or immunoblot that the specific genes or encoded proteins are downregulated in the shRNA-CaP cells compared to their parental counterparts.

We are currently pursuing this aim. shRNA clones with increased invasive potentials have been isolated and are being expanded for bar-code-specific PCR.

Task 9. Demonstrate that re-expression of an identified gene can suppress CaP metastatic potential. (Months 15-17).

-transiently express a full-length cDNA of at least one gene identified in *Task 8* into the metastatic human CaP cells, PC-3M and DU145. Determine whether the gene expression inhibits Matrigel invasiveness compared to parental cells transfected with empty expression vector alone.

We will begin this Task after identifying the shRNA-mediated gene knockdowns in our CaP clones (within the next 4 months).

Task 10. Correlate metastasis-gene downregulation with increased CaP Gleason sum or metastatic potential. (Months 15-17).

-perform *in situ* hybridization of CaP tumor microarrays (commercial and those produced in RPCI) using anti-sense (or as negative controls, sense) cRNA probes, or alternatively, if appropriate antibodies are available (i.e.- they recognize only the specific proteins on SDS-PAGE analysis and they work in formalin-fixed tissues), perform immunohistochemistry on the TMAs. Correlate loss of gene/protein signal with increasing Gleason sum, or clinical note of disease recurrence (i.e.- appearance of androgen-independent disease) or appearance of bone metastases. Note that this part of the study is an initial analysis and thus, although it may not satisfy sufficient power analysis, it may show trends of gene/protein loss with increased disease progression.

We will begin this Task after identifying the shRNA-mediated gene knockdowns in our CaP clones and then procuring appropriate Ab reagents (within the next 8 months). In anticipation, we are working with Carl Morrison, DVM PhD, to use a newly-produced tissue microarray containing 732 CaP cases (plus, in many cases, matched normal tissue controls) with known clinical outcomes reflecting all CaP cases at RPCI since 1993.

Task 11. Prepare report. (Months 17-18).

Completed here.

KEY RESEARCH ACCOMPLISHMENTS

- successful transduced LNCaP and LAPC-4 CaP cells with shRNAs, using our cancer gene-specific retrovirus shRNA sub-library and our GIPZ lentivirus human genomic library.
- successful selection (puromycin resistance for the retrovirus clones, GFP expression for the GIPZ clones) and expansion of these CaP cells *in vitro*.
- demonstration that the shRNA-infected (vs. empty vector) cells exhibited greatly enhanced invasive potentials over control cells.
- isolation of individual shRNA-expressing CaP clones for bar-code-specific PCR identification of knocked down genes.
- established collaborators with technical experience in i.c. injection and isolation of CaP cells from femur bone marrows.
- established pools of shRNA-infected CaP cells to be used in i.c. injections of nude mice.

REPORTABLE OUTCOMES

- isolation and validation of a cancer-specific retrovirus shRNA library and a human genomic shRNA library in lentiviruses (GIPZ).
- isolation of LNCaP and LAPC-4 cell pools with stably transduced genomic shRNAs.
- isolation of LNCaP and LAPC-4 cells with increased spontaneous Matrigel invasiveness, and with even greater invasive potential after shRNA transduction.
- isolation of individual shRNA-infected and spontaneous CaP clones exhibiting enhanced invasiveness.

CONCLUSION

We have successfully produced human CaP clones that are stably transduced with shRNAs clones representing the entire human genome. We also isolated CaP clones with increased spontaneous and shRNA-induced invasive potentials using *in vitro* selection systems. We are in the process of identifying and validating the individual genes whose knockdown potentially increases CaP invasiveness. Given that our data indicate greatly increased invasive potentials in the shRNA-infected clones over controls, there is confidence that these transduced pools will also yield increased rates of bone colonization and bone tumor growth.

REFERENCES

None

APPENDICES

None